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Overexpression of circadian clock protein cryptochrome (CRY) 1 alleviates sleep deprivation-induced vascular inflammation in a mouse model

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ABSTRACT

Disturbance of the circadian clock by sleep deprivation has been proposed to be involved in the regulation of inflammation. However, the underlying mechanism of circadian oscillator components in regulating the pro-inflammatory process during sleep deprivation remains poorly understood. Using a sleep deprivation mouse model, we showed here that sleep deprivation increased the expression of pro-inflammatory cytokines expression and decreased the expression of cryptochrome 1 (CRY1) in vascular endothelial cells. Furthermore, the adhesion molecules including intercellular adhesion molecule-1, vascular cell adhesion molecule-1 and E-selectin were elevated in vascular endothelial cells and the monocytes binding to vascular endothelial cells were also increased by sleep deprivation. Interestingly, overexpression of CRY1 in a mouse model by adenovirus vector significantly inhibited the expression of inflammatory cytokines and adhesion molecules, and NF–κB signal pathway activation, as well as the binding of monocytes to vascular endothelial cells. Using a luciferase reporter assay, we found that CRY1 could repress the transcriptional activity of nuclear factor (NF)–κB in vitro. Subsequently, we demonstrated that overexpression of CRY1 inhibited the basal concentration of cyclic adenosine monophosphate (cAMP), leading to decreased protein kinase A activity, which resulted in decreased phosphorylation of p65. Taken together, these results suggested that the overexpression of CRY1 inhibited sleep deprivation-induced vascular inflammation that might be associated with NF–κB and cAMP/PKA pathways.

1. Introduction

In modern society, sleep loss, such as sleep deprivation and insomnia, has become more and more prevalent, especially in shift-workers [1,2]. The deficiency of sleep not only leads to increased amounts of accidents at work, but also causes considerable damage to human health [3]. A number of physiological and behavioral functions including hormone secretion, cognitive ability, memory and immune function have been indicated to be affected by sleep deprivation [4,5]. Notably, sleep deprivation has been proposed to be associated with high risk of numerous diseases including cancer, autoimmune diseases and cardiovascular diseases [6–10]. Therefore, it is of particular importance to understand the potential mechanism by which sleep deprivation influences human health.

Previous studies have demonstrated that sleep deprivation affects inflammation [11]. Numerous pro-inflammatory cytokines, interleukin (IL)-1, IL-6 and tumor necrosis factor (TNF), have been reported to be increased by sleep deprivation [12–15]. C-reactive protein, a critical marker of cardiovascular risk [16], is found to be elevated in people suffering sleep deprivation [10]. Endothelial dysfunction plays an important role in the initiation and development of cardiovascular disease, especially arteriosclerosis, which is characterized by the inflammation of vascular endothelial cells [17]. Sleep deprivation has been reported to trigger endothelial dysfunction [18–20]. Sauvet et al. [21] demonstrated that serum levels of IL-6 and adhesion molecules, including intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin, were elevated by sleep deprivation. However, limited studies have focused on the effect of sleep deprivation on

Abbreviations: CRY, cryptochrome; IL, interleukin; TNF, tumor necrosis factor; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; NF–κB, nuclear factor; cAMP, cyclic adenosine monophosphate; CLOCK, transcriptional activators circadian locomotor output cycles kaput; BMAL, brain and muscle aryl hydrocarbon receptor nuclear translocator-like 1; PER, Period; VASP, vasodilator-stimulated phosphoprotein.

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vascular inflammation; the underlying mechanism is poorly understood.

The circadian clock is an important physiological process for maintaining homeostasis [22]. Circadian rhythm is constructed by a group of clock genes, including transcriptional activators circadian locomotor output cycles kaput (CLOCK), brain and muscle aryl hydrocarbon receptor nuclear translocator (ARNT)-like (BMAL)1, Period (PER) and Cryptochrome (CRY) [23]. The circadian rhythmicity is maintained in a transcription–translation feedback manner in which the CLOCK/BMAL complex activates the expression of PER and CRY, which, in turn, inhibit activity of the CLOCK/BMAL complex [24]. Most recently, it has been intriguingly reported that the arrhythmic clock system has a molecular link with inflammation. The lack of CRY, the core clock component, has been demonstrated to lead to increased pro-inflammatory cytokine activation via the nuclear factor (NF)–κB pathway [25]. The deletion of CRY1 and CRY2 aggravates arthritis via the regulation of various cytokines including IL-1β, IL-6 and TNF-α, whereas the overexpression of CRY1 significantly decreased the activation of TNF-α gene expression [26].

To date, whether the CRY-mediated inflammation is involved in vascular inflammation during sleep deprivation remains poorly understood. In the present study, we investigated the effect of sleep deprivation on the expression of pro-inflammatory cytokines and adhesion molecules in vascular endothelial cells. We found that CRY1 expression was significantly reduced during sleep deprivation in an animal model, while the overexpression of CRY1 alleviated sleep deprivation-induced vascular inflammation associated with NF–κB and PKA signaling pathway. Our study provided a possible molecular link between sleep deprivation and vascular inflammation through which sleep deprivation might increase the risk of cardiovascular disease. Moreover, we have identified that CRY1 might be a novel effector molecular target for interventional therapy of sleep deprivation-induced diseases.

2. Materials and methods

2.1. Animals

A total of fifteen eight-week-old male C57BL/6 mice (weighing 20–25 g) were purchased from Changzhou Cavens Laboratory Animal Co., Ltd. (Changzhou, Jiangsu, China). Mice were housed under standard conditions of room temperature, humidity and dark-light cycles in pathogen-free microisolator cages with free access to water and food. The animal experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Central South University.

2.2. Cell cultures

Mouse aortic endothelial cells were isolated from the thoracic aortas according to a previously reported method with minimal modifications [27]. Briefly, mice were anesthetized by the subcutaneous injection of sodium pentobarbital (40 mg/kg). After cleaning with 70% ethanol, the thoracic cavities were opened and thoracic aortas were isolated and perfused with Hank's buffered salt solution containing 0.5% Triton X-100. The aorta was then placed into Dulbecco's Modified Eagle Medium (DMEM) with collagenase type 2 (300 U/ml) in a petri dish. The aorta open walls were then softly scraped with a razor blade. Thereafter, the mixture was immediately incubated at 37 °C in a water bath for 50 min. The cells were collected in a sterile manner using 100-μm cell strainer (BD Biosciences, San Jose, CA, USA). The collected cells were then resuspended with pre-warmed DMEM containing 20% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) followed by centrifugation at 2000 rpm for 8 min at 4 °C. The pellet was collected and resuspended with 1 ml Enzyme-Free Cell Dissociation Solution (Sciencell, Carlsbad, CA, USA) supplemented with 35-μl magnetic beads coated with anti-CD146 antibody (Millipore, Temecula, CA, USA). The mixtures were incubated and rocked at 4 °C for 30 min. Then, the beads were washed with ice-cold ECDS on a magnetic rack three times, followed by washing with ice-cold HEPES-buffered saline solution (Lonza, Walkersville, MD, USA) once. The supernatant was removed and the remaining magnetic beads coated with cells were incubated with trypsin at 37 °C for 10 min. The mixture, in the tube, was placed back on the magnetic rack and the supernatant containing endothelial cells were collected and grown in DMEM with 10% FBS, 1% endothelial growth supplement and 1% penicillin and streptomycin. Mouse macrophage RAW264.7 cells purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) were grown in DMEM supplemented with 1 mM sodium pyruvate, 10% FBS and 1% of penicillin and streptomycin at 37 °C in a 5% CO_2 chamber.

2.3. Sleep deprivation and experimental design

Sleep deprivation of mice was established using the classical platform technique [28]. Briefly, mice (five in a group) were placed on a narrow platform in a water tank. About 14 platforms (3 cm in diameter) were in a water tank about 4 cm above the surface of the water. The mice could move from one platform to another in the water tank by jumping. In the experiment, when the mice slept, they would fall into the water due to muscle atonia induced by sleep. Therefore, the mice had to wake up and climb up the platform. The duration of sleep deprivation was 20 h, which began from 2 am to 10 pm over 7 days. On the other hand, the mice had 4 h of sleep from 10 ante-meridiem to 2 post-meridiem. For this experiment, a total of fifteen mice were randomly divided into three groups. Control group mice were placed on a large platform in the water tank where the mice were able to sleep and move at will. The SD + GFP group was the group of sleep deprivation mice which were injected with GFP-adenovirus vector by (1×10^8 pfu) tail-vein injection. The SD + CRY1 group contained sleep deprivation mice that were injected with CRY1-expressing adenovirus vector (1×10^8 pfu) (Biowit, Shenzhen, China). At the end of the experiment, all mice were euthanized by the subcutaneous injection of sodium pentobarbital (40 mg/kg).

2.4. Monocyte adhesion assay

The in vitro monocyte adhesion assay was conducted according to a previously reported method [29]. Briefly, mouse aortic endothelial cells grown in 48-well plates were pretreated with adenovirus vector for 24 h. Then, TNFα (2 ng/ml; R&D Systems, Inc., Minneapolis, MN, USA) was added and incubated for 6 h. Thereafter, mouse aortic endothelial cells were washed with serum-free medium labeled with calcein-AM (DMEM medium containing 1% FBS) and were then incubated for 1 h followed by washing with PBS to remove unbound monocytes. Images were captured using a fluorescence microscope. For the ex vivo monocyte adhesion assay, mice were euthanized and thoracic aortas were rapidly excised. The aortas were trimmed to remove connective tissue and fat, followed by washing with ice-cold PBS. Thereafter, the aortas were placed into DMEM and incubated for 10 min at 37 °C. The endothelia were exposed and pinned onto a slide glass with 4% agar and 1 ml of DMEM containing 1% heat-inactivated FBS. About 1×10^6/ml RAW264.7 cells labeled with calcein-AM were incubated with the above aortas in DMEM medium containing 1% FBS for 1 h. The unbound monocytes were then washed away by PBS and

the bound monocytes were detected and captured using a confocal microscopy.

2.5. Quantitative real-time polymerase chain reaction (qRT-PCR)

Mouse aortic endothelial cells from mice were collected in cell lysis buffer. Total RNA was extracted from cells using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the supplier's instructions. Reverse transcription of 5 μg of the total RNA into cDNA was performed by using M-MLV reverse transcriptase (Clontech, Palo Alto, CA, USA). For the measurement of gene expression, a total of 10 μl mixture containing 1 μl of cDNA, 2 μl of each of the forward and reverse primers (1 μM) and 5 μl SsoFast™ EvaGreen Supermix (Bio-Rad, Hercules, CA, USA) was used for the PCR procedure as follows: initial denaturation at 94 °C for 5 min; 30 cycles of denaturation at 94 °C for 20 s, annealing at 55 °C for 30 s and extension at 72 °C for 20 s; and melt curve from 65 to 95 °C. GAPDH was used as the internal reference gene. The relative expression levels were calculated by $2^{-\Delta\Delta Ct}$ method and the target gene was normalized to the internal reference gene.

2.6. Western blot analysis

The protein in cell lysis was separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by transference to a nitrocellulose membrane (Amersham, Little Chalfont, UK). The membrane was then blocked with 2.5% nonfat dry milk for 1 h. Primary antibodies including anti-IL-1β (sc-7884), anti-IL-6 (sc-1266), anti-TNF-α (sc-1351), anti-ICAM-1 (sc-1511), anti-VCAM-1 (sc-1504), E-selectin (sc-14011), and anti-GAPDH (sc-20357) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); phosphor-p65 (3037) and phosphor-VASP (3111) were purchased from Cell Signaling Technology (Danvers, MA, USA). These antibodies were added and incubated overnight at 4 °C. After incubation with the corresponding horseradish peroxidase-conjugated secondary antibody (Boster Corporation, Wuhan, China), the target protein was visualized by enhanced chemiluminescence (Pierce, Rockford, IL, USA).

2.7. Luciferase reporter assay

Mouse aortic endothelial cells seeded in 24-well plates were co-transfected with NF–κB promoter-luciferase vector (Promega, Madison, WI, USA) and adenovirus vector expression GFP or CRY1. The transfected cells were cultured for 48 h before the addition of 2 ng/ml TNF-α for an additional incubation for 6 h. Then, the cells were washed with PBS and lysed in a reporter lysis reagent. The luciferase activity in cell extracts was measured using the dual-luciferase reporter assay system (Promega, Madison, WI, USA).

2.8. Intracellular cAMP assay

Mouse aortic endothelial cells were harvested from mice and the intracellular cAMP levels were measured by an EIA cAMP ELISA kit (Assay Designs, Ann Arbor, MI, USA), according to the manufacturer's instructions.

2.9. Statistical analysis

The data were expressed as mean ± standard deviation (SD). The significance of variability between two groups or among multiple groups was determined by Student's *t*-test and one-way analysis of variance (ANOVA) followed by Bonferroni's test, respectively. A *p* value of less than 0.05 was considered statistically significant.

3. Results

3.1. Sleep deprivation induces vascular inflammation and suppresses CRY1 expression

Sleep deprivation has been considered to be related to the increased levels of cardiovascular disease [30]. Vascular endothelial dysfunction is an early predictor of many cardiovascular diseases, including atherosclerosis [17]. To date, limited studies have focused on the effect of sleep deprivation on vascular function [21,31]; therefore, the precise biological mechanism remains largely unknown. In the present study, we used a mouse model of sleep deprivation to explore the biological effect of sleep deprivation on vascular inflammation, which plays an important role in the initiation of atherosclerosis [32]. We first determined the expression patterns of pro-inflammatory cytokines in mouse aortic endothelial cells from the sleep deprivation group. Significantly increased mRNA (Fig. 1A–C) and protein (Fig. 1D–G) expression levels of IL-1β, IL-6 and TNF-α were observed in sleep deprivation mice relative to control mice. We also found that the mRNA (Fig. 1H) and protein (Fig. 1) expression of CRY1, a key circadian clock component, was significantly down-regulated in sleep deprivation mice compared with control mice.

3.2. Overexpression of CRY1 inhibits sleep deprivation-induced vascular inflammation

To investigate whether CRY1 was involved in the regulation of vascular inflammation during sleep deprivation, we overexpressed CRY1 in sleep deprivation mice by the infection of recombinant adenovirus vector via tail vein injection and the expression of CRY1 in different groups were detected by Western blot analysis (Fig. 2A). The results showed that Ad-CRY1 infected groups showed significant elevated expression levels of CRY1 in comparison with Ad-GFP infected groups (Fig. 2B). Overexpression of CRY1 significantly down-regulated both the mRNA (Fig. 2C–E) and protein (Fig. 2F–I) expression levels of IL-1β, IL-6 and TNF-α, which were induced by sleep deprivation in vascular endothelial cells. These data suggested that CRY1 had a regulatory effect on sleep deprivation-induced pro-inflammatory cytokine expression.

3.3. Overexpression of CRY1 inhibits the expression of adhesion molecules in vascular endothelial cells

The accumulation of monocytes in the vascular wall of arteries is an important event for the initiation and development of atherosclerosis, where the inflammation activation of vascular endothelial cells triggered by different factors such as inflammatory cytokines and TNF-α plays a critical role [33,34]. To explore whether sleep deprivation altered the expression of adhesion molecules in vascular endothelial cells, we analyzed the expression patterns of adhesion molecules including VCAM-1, ICAM-1 and E-selectin. We found that the mRNA expression of VCAM-1, ICAM-1 and E-selectin in vascular endothelial cells was significantly increased by sleep deprivation. In contrast, the increased mRNA expression of VCAM-1 (Fig. 3A), ICAM-1 (Fig. 3B) and E-selectin (Fig. 3C) was reversed in sleep deprivation mice infected with CRY1-overexpressing adenovirus vectors. The expression levels of VCAM-1, ICAM-1 and E-selectin were further examined by Western blot analysis (Fig. 3D). Western blot results demonstrated that the protein expression of VCAM-1, ICAM-1 and E-selectin elevated by sleep deprivation was also apparently decreased in sleep deprivation mice infected with CRY1-overexpressing adenovirus vectors (Fig. 3E–G). These results implied that sleep deprivation

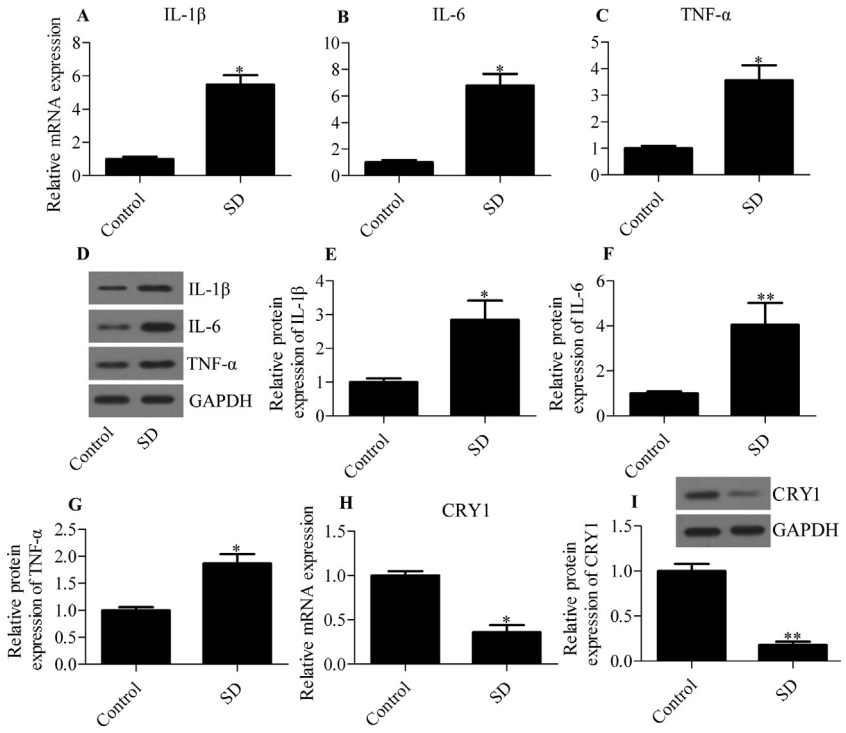

Fig. 1. Effect of sleep deprivation on vascular inflammation in mouse models. mRNA expression levels of IL-1β (A), IL-6 (B) and TNF-α (C) in mouse aortic endothelial cells from a mouse model was detected by qRT-PCR. The fold changes of mRNA were presented after normalization with control group. Data were expressed as mean ± SD from independent mice ($n = 5$), $^{*}p < 0.05$ vs. control. (D) Western blot analysis of IL-1β, IL-6 and TNF-α by the antibodies indicated. Relative protein levels of IL-1β (E), IL-6 (F) and TNF-α (G) were quantified using Image-Pro Plus 6.0 software and normalized to GAPDH. GAPDH was used as the internal control. Data were expressed as mean ± SD from independent mice ($n = 3$), $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control. (H) mRNA expression level of CRY1 was analyzed by qRT-PCR. (I) Protein expression level of CRY1 was detected by Western blot analysis with indicated antibodies, and quantified using Image-Pro Plus 6.0 software. Data were expressed as mean ± SD from independent mice ($n = 3$), $^{**}p < 0.01$ vs. control.

increased the expression of adhesion molecules which could be inhibited by CRY1 overexpression.

3.4. Overexpression of CRY1 suppresses the binding of monocytes to endothelial cells

Because sleep deprivation induced the expression of adhesion molecules in vascular endothelial cells, we speculated that sleep deprivation might increase monocyte binding to the endothelium of aortic vessels of mice in vivo. To test this hypothesis, we analyzed the effect of sleep deprivation on monocyte binding to the endothelium in mouse aortic vessels in vivo. The ex vivo monocyte adhesion assay showed that the mouse aortic endothelium isolated from sleep deprivation mice had higher binding to mouse macrophages, whereas overexpression of CRY1 significantly inhibited the binding activity of aortic endothelium isolated from sleep deprivation mice to mouse macrophages (Fig. 4A and B). To further verify the inhibitory function of CRY1 on monocyte binding, we determined the effect of CRY1 on TNF-α-induced monocyte binding in endothelial cells in vitro. The results showed that the overexpression of CRY1 in endothelial cells markedly suppressed TNF-α-induced monocyte binding (Fig. 4C and D).

3.5. Overexpression of CRY1 inhibits the activation of NF–κB

Extensive evidence has demonstrated that NF–κB is essential for transcriptional regulation and the expression of inflammatory cytokines and adhesion molecules in endothelial cells [35,36]. Translocation of the NF–κB p65 subunit has been reported to exert important roles in the intimal thickening of endothelial cells [37]. The phosphorylation of p65 is essential for its nuclear retention and transcriptional activity [38]. For analysis of the activation of NF–κB in aortic endothelial cells of sleep deprivation mice, we detected the phosphorylation status of p65 using Western blot analysis (Fig. 5A). The results showed that the phosphorylation of p65 was significantly up-regulated in endothelial cells by sleep deprivation compared with the control group. However, the facilitating effect of sleep deprivation on the phosphorylation of p65 was markedly inhibited by CRY1 overexpression in mice (Fig. 5B). However, the total expression level of p65 was not apparently affected by sleep deprivation or CRY1 overexpression (Fig. 5C). To further verify the regulatory effect of CRY1 on NF–κB, we examined the effect of CRY1 overexpression on the TNF-α-induced NF–κB activation in endothelial cells by luciferase reporter assay. The results showed that the TNF-α-treated endothelial cells had potently increased NF–κB transcriptional activity compared with non-treated cells. In contrast, the overexpression of CRY1 significantly inhibited TNF-α-induced NF–κB transcriptional activity in endothelial cells (Fig. 5D). In summary, these findings suggested that CRY1 overexpression suppressed the activation of NF–κB in endothelial cells in vivo or in vitro.

3.6. Overexpression of CRY1 increases cAMP levels and PKA activity

The PKA activation caused by high levels of cAMP contributes to the activation of NF–κB due to the ability of PKA to phosphorylate p65 at Ser276 [39,40]. In the endothelial cells of sleep-deprived mice, we found that the basal concentration of cAMP was significantly increased in comparison with those in the control group (Fig. 6A). Meanwhile, the activity of PKA, indicated by the phosphorylation of vasodilatator-stimulated phosphoprotein (VASP), a known substrate of PKA [41], was also significantly increased in sleep-deprived mice (Fig. 6B and C). However, these effects were

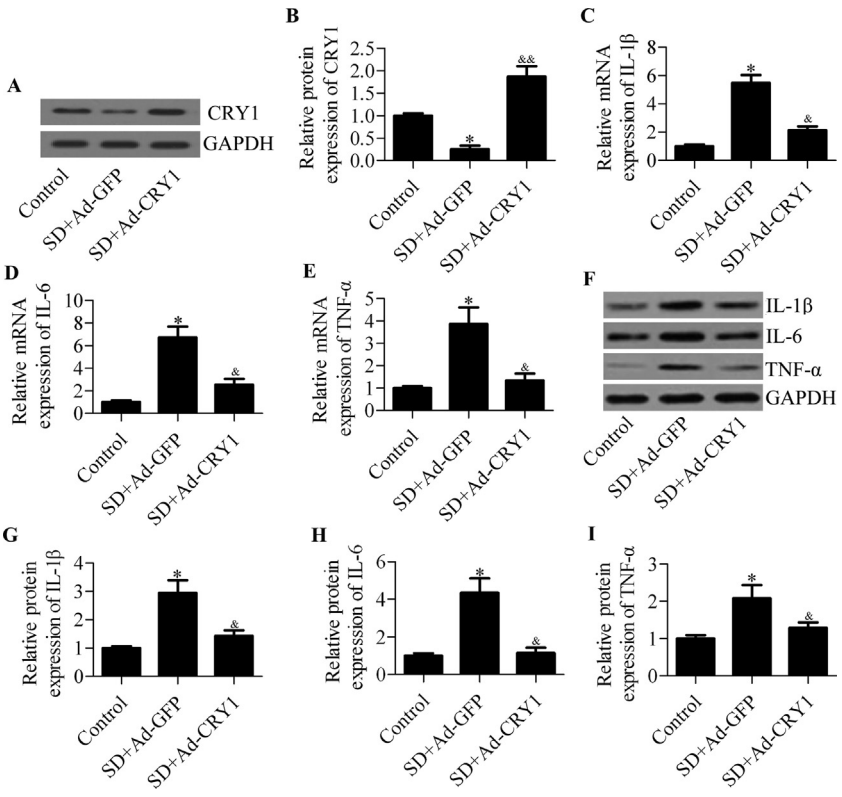

Fig. 2. Effect of CRY1 overexpression on sleep deprivation-induced vascular inflammation. (A) Western blot analysis to detect the protein expression levels of CRY1 vascular endothelial cells in different treated groups, and relative protein levels was quantified using Image-Pro Plus 6.0 software. The fold changes were presented after normalization with control group. Data were expressed as mean ± SD from independent mice ($n = 3$), $^{*}p < 0.05$ vs. control and $\&\&p < 0.01$ vs. SD + Ad-GFP. Control, mice without treatment; SD + Ad-GFP, mice were treated with sleep deprivation and received infection of an adenovirus vector carrying GFP; SD + Ad-CRY1, mice were treated with sleep deprivation and received infection of adenovirus vector carrying CRY1. mRNA expression levels of IL-1β (C), IL-6 (D) and TNF-α (E) in vascular endothelial cells were determined by qRT-PCR analysis. Data were expressed as mean ± SD from independent mice ($n = 5$), $^{*}p < 0.05$ vs. control and & <0.05 vs. SD + Ad-GFP. (F) The protein expression levels of IL-1β, IL-6 and TNF-α in vascular endothelial cells were detected by Western blot analysis using the antibodies indicated. The relative protein expression levels of IL-1β (G), IL-6 (H) and TNF-α (I) were quantified using Image-Pro Plus 6.0 software and normalized to GAPDH. Data were expressed as mean ± SD from independent mice ($n = 3$), $^{*}p < 0.05$ vs. control and & <0.05 vs. SD + Ad-GFP.

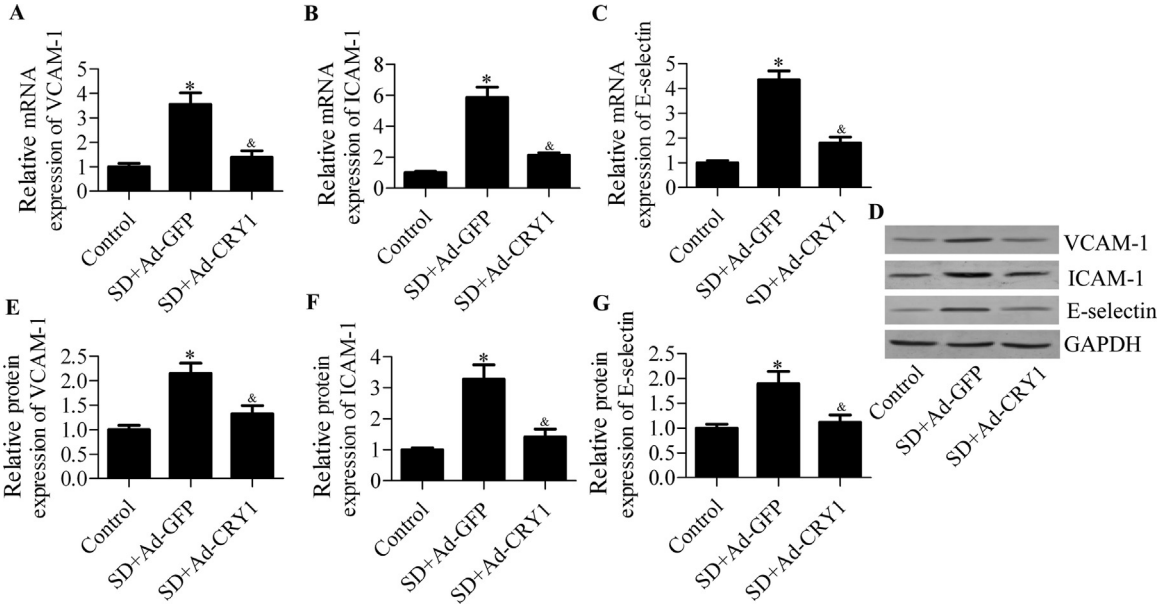

Fig. 3. Effect of sleep deprivation and CRY1 on the expression of adhesion molecules. mRNA expression levels of adhesion molecules including VCAM-1 (A), ICAM-1 (B) and E-selectin (C) in endothelial cells were analyzed by qRT-PCR. Data were expressed as mean ± SD from independent mice ($n = 5$), $^{*}p < 0.05$ vs. control and & <0.05 vs. SD + Ad-GFP. (D) Western blot analysis was performed to detect the protein expression levels of adhesion molecules including VCAM-1, ICAM-1 and E-selectin using the antibodies indicated. The relative protein expression levels of VCAM-1 (E), ICAM-1 (F) and E-selectin (G) were quantified using Image-Pro Plus 6.0 software and normalized to GAPDH. Data were expressed as mean ± SD from independent mice ($n = 3$), $^{*}p < 0.05$ vs. control and & <0.05 vs. SD + Ad-GFP.

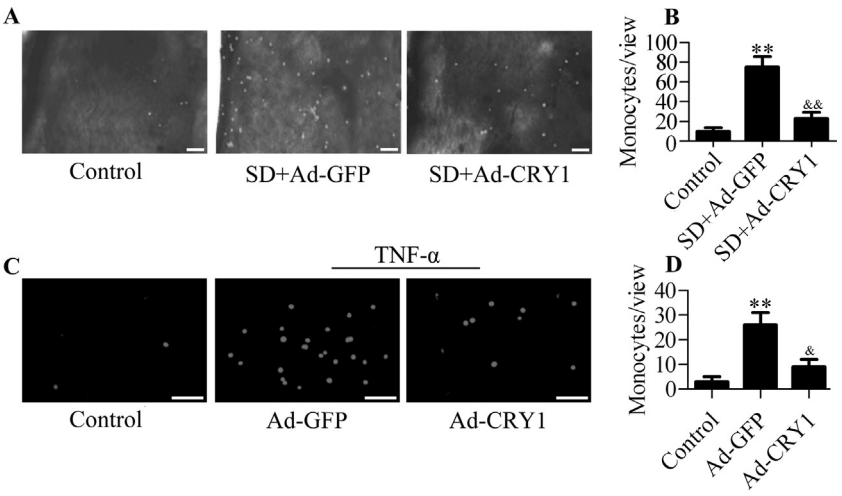

Fig. 4. Effect of CRY1 overexpression on the binding of monocytes to endothelial cells. (A) The monocyte binding to aortic endothelium was determined by an ex vivo monocyte adhesion assay. The endothelium was isolated from the control group, SD + Ad-GFP group or SD + Ad-CRY1 group, and incubated with mouse macrophages labeled with calcein-AM. The scare bar denotes 200 μm at 100× magnifications. (B) Representative histograms show the number of positive adhesion monocytes binding to aortic endothelium in different groups. Data were expressed as mean ± SD from independent mice ($n = 5$), $^{**}p < 0.01$ vs. control, and && <0.01 vs. SD + Ad-GFP. (C) An in vitro monocyte adhesion assay was performed to detect the binding of monocytes to endothelial cells. The cultured mouse aortic endothelial cells were pre-infected with adenovirus vector for 24 h before TNF-α (2 ng/ml) was added and incubated for 6 h. Cells without treatment were used as a control. Then, cells were incubated with mouse macrophages labeled with calcein-AM. The scare bar denotes 200 μm at 100× magnifications. (D) Representative histograms show the number of positive adhesion monocytes binding to endothelial cells in different groups. Data were expressed as mean ± SD from independent mice ($n = 5$), $^{**}p < 0.01$ vs. control, and & <0.05 vs. Ad-GFP.

reversed in CRY1 overexpressed mice. The total protein level of VASP was not altered by sleep deprivation or CRY1 overexpression (Fig. 6D). The data suggest that CRY1 overexpression suppresses cAMP production and PKA activation.

4. Discussion

In the present study, we provided evidence that sleep deprivation increased vascular inflammation and the binding of monocytes to vascular endothelial cells, all of which contributes to the initiation and development of atherosclerosis. We further demonstrated that the core clock gene CRY1 plays an important role in the triggering of endothelial dysfunction through regulating inflammatory processes during sleep deprivation, implying a possible molecular link between the arrhythmic clock system and vascular inflammation.

Increasing evidence has demonstrated that sleep deprivation induces inflammatory responses with the increased expression

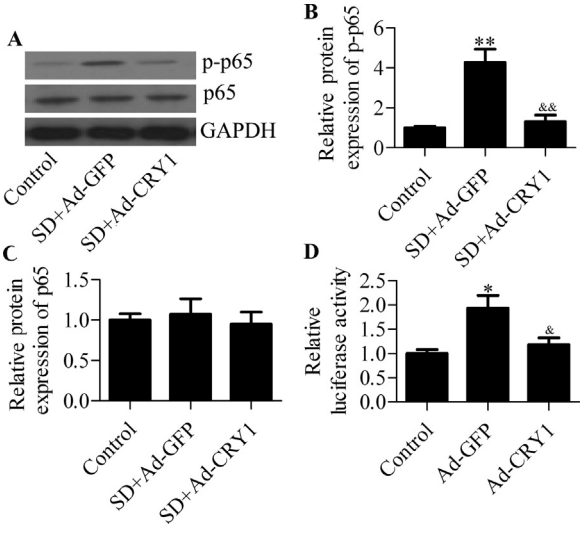

Fig. 5. Effect of CRY1 overexpression on NF–κB. (A) Western blot to detect the level of phospho-p65 (p-p65) in endothelial cells isolated from control, SD + Ad-GFP or SD + Ad-CRY1 mice. Quantitative analysis of protein expression levels of p-p65 (B) and total p65 (C) using Image-Pro Plus 6.0 software and normalized to GAPDH. Data were expressed as mean ± SD from independent mice ($n = 3$), $^{**}p < 0.01$ vs. control and && <0.01 vs. SD + Ad-GFP. (D) The effect of CRY1 on TNF-α-induced NF–κB transcriptional activity. Mouse aortic endothelial cells were co-transfected with NF–κB promoter-luciferase vector and adenovirus vector for 14 h. Then, TNF-α (2 ng/ml) was added and incubated for an additional 6 h. The luciferase activity in cell extracts was measured using the dual-luciferase reporter assay kit. Data were expressed as mean ± SD ($n = 3$), $^{*}p < 0.05$ vs. control and & <0.05 vs. Ad-GFP.

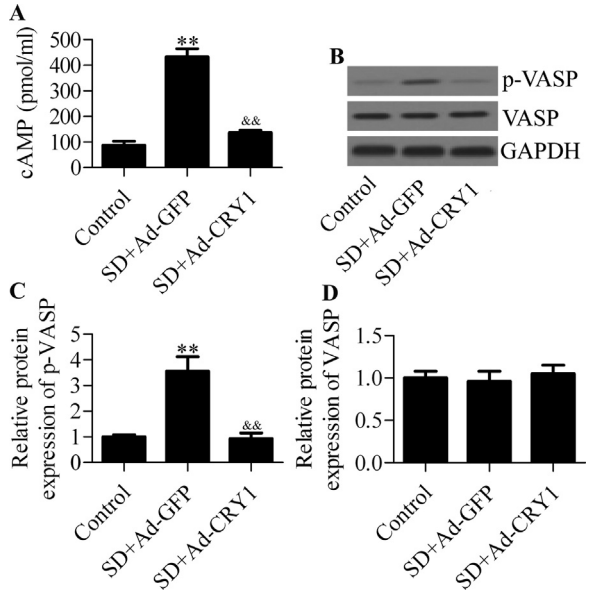

Fig. 6. Effect of CRY1 overexpression on cAMP levels and PKA activity. (A) Cellular concentration of cAMP was measured by a cAMP ELISA kit in the lysates of endothelial cells from control, SD + GFP or SD + Ad-CRY1 mice. Data were expressed as mean ± SD from independent mice ($n = 3$), $^{**}p < 0.01$ vs. control and && <0.01 vs. SD + Ad-GFP. (B) The levels of phospho-VASP (p-VASP) and VASP in endothelial cells isolated from different groups were detected by Western blot analysis with the antibodies indicated. Quantitative analysis of protein expression levels of p-VASP (C) and total VASP (D) using Image-Pro Plus 6.0 software and normalized to GAPDH. Data were expressed as mean ± SD from independent mice ($n = 3$), $^{**}p < 0.01$ vs. control and && <0.01 vs. SD + Ad-GFP.

of serum pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α in both animal and human models [15,42–44]. The NF–κB signaling pathway which regulates the transcriptional expression of various inflammatory cytokines, contributing to the pathophysiology of atherosclerosis, was also found to be activated in peripheral blood mononuclear cells by sleep deprivation [45,46]. Currently, increasing studies have focused on the sleep deprivation-involved inflammatory bowel disease. Tang et al. [47] revealed that sleep deprivation promotes colonic inflammation, which aggravates colitis in a mouse model. Most recently, Chung et al. demonstrated that sleep deprivation worsens the inflammation of colitis in mice, which could be improved by melatonin as it functions as a mediator of circadian rhythms [48,49]. Similarly, recent studies have reported that melatonin protects against lung inflammation associated with sleep deprivation [50,51]. It is interesting that sleep deprivation has been reported to have an anti-inflammatory effect during ischemic cell death or lipopolysaccharide infection, in which sleep deprivation was considered a preconditioning stimulus that provides neuroprotection [52]. In the present study, we found that sleep deprivation increased vascular inflammation through up-regulating pro-inflammatory cytokines, including IL-1β, IL-6 and TNF-α, in endothelial cells. Our data indicated that sleep deprivation was involved in pro-inflammation through the NF–κB signaling pathway. Our findings provided novel insights into the sleep deprivation-induced high risk for cardiovascular disease, which is linked with vascular inflammation.

In the current study, we also found that sleep deprivation increased the expression of adhesion molecules including VCAM-1, ICAM-1 and E-selectin, which increased monocyte binding to endothelial cells. The accumulation of monocytes in the vascular wall of arteries is an important event for the initiation and development of atherosclerosis [33,34]. Therefore, the accumulation of monocytes induced by sleep deprivation in the vascular wall increased the risk for the development of atherosclerosis. Our findings were consistent with the report of Sauvet et al., which revealed that sleep deprivation increased the concentration of serum adhesion molecules in healthy subjects [21]. Van Leeuwen et al. [53] demonstrated that sleep deprivation increases lymphocyte activation and the expression of pro-inflammatory cytokines including IL-1β, IL-6, IL-17 and C reactive protein, which augments the risk of developing cardiovascular diseases. Interestingly, our data demonstrated that sleep deprivation-induced adhesion molecules expression was reversed by CRY1 overexpression, implying that CRY1 might be involved in regulating proinflammatory cytokines and chemokines expression. Recently, the inflammatory monocyte chemokine, chemokine (C-C motif) ligand 2 (CCL2) has also been reported to be regulated by circadian clock and to mediate a circadian control of monocyte/macrophage recruitment. Induction of MAL1:CLOCK heterodimer has been shown to inhibit the expression of CCL2 and IL-6 [54]. It has been recently revealed that overexpression of Rev-erbα, a circadian clock gene, suppresses CCL2 expression and impairs cell adhesion and migration of murine macrophage [55]. Sugimoto et al. [56] reported that knockdown of Period1 (Per1), one of the clock genes, led to an increase of the expression of CCL2 and IL-6 in cultured rat spinal astrocytes associated with the activation of p38, c-Jun N-terminal kinase (JNK), and NF–κB pathways. In the present study, our data suggested that CRY1 overexpression inhibited the expression of pro-inflammatory cytokines and adhesion molecules. However, whether CRY1 plays a role in regulating CCL2 expression during sleep deprivation needs to be delineated in future studies.

In the present study, we found that CRY1 was decreased in sleep-deprived mice, and the overexpression of CRY1 could inhibited vascular inflammation induced by sleep deprivation. Our data also suggested that CRY1 overexpression affected NF–κB pathway which was a critical pathway regulating the inflammatory process. Several studies have revealed the role of CRY in the regulation of inflammation. A lack of CRY, the core clock component which comprises CRY1 and CRY2, has been reported to lead to increased pro-inflammatory cytokine activation through the NF–κB pathway [25]. The deletion of CRY1 and CRY2 aggravates arthritis via the regulation of various cytokines including IL-1β, IL-6 and TNF-α, whereas the overexpression of CRY1 significantly decreased the activation of TNF-α gene expression [26]. Moreover, our data also suggested that overexpression of CRY1 inhibited cAMP/PKA. We found that overexpression of CRY1 significantly inhibited the basal concentrations of cAMP in endothelial cells and resulted in inactivation of PKA. Overexpression of CRY1 has been demonstrated to suppress the production of cAMP by interacting with adenylyl cyclase [25]. Zhang et al. [57] reported that the overexpression of CRY1 inhibited the G protein-coupled receptor (GPCR) activation-dependent cAMP production through interaction with Gsα. PKA activation caused by high levels of cAMP has been clearly recognized to activate NF–κB through phosphorylation of the NF–κB subunit p65 at Ser276 [39,40]. Therefore, it is possible that sleep deprivation suppresses CRY1 expression which may be associated with cAMP/PKA and NF-κB pathways that contribute to vascular inflammation. However, further studies are required to delineate the precise mechanism of CRY1 in regulating vascular inflammation.

In conclusion, we demonstrated that sleep deprivation increased the vascular inflammation and endothelial dysfunction through affecting CRY1, which might be associated with the high risk of atherosclerosis. We found that CRY1 was decreased in sleep-deprived mice, and overexpression of CRY1 alleviated sleep deprivation-induced vascular inflammation, implicating CRY1 as a novel and effective molecular target for the interventional therapy of sleep deprivation-induced diseases. However, the limitations of our study should be considered. We only focused on CRY1, whereas the effect of sleep deprivation on CRY2 and the effect of CRY2 on inflammation induced by sleep deprivation were not investigated. Considering the role of CRY in the regulation of circadian rhythmicity, one can speculate that CRY2 should also be involved in sleep deprivation. In the present study, our data demonstrated that overexpression of the sole member of CRY, CRY1, could effectively reverse the sleep deprivation-induced inflammation in vascular endothelial cells. Nonetheless, whether CRY2 plays a role in regulating sleep deprivation-induced vascular inflammation remains unknown. Further studies are required to fully understand the underlying mechanism of CRY in regulating vascular inflammation during sleep deprivation.

Conflict of interests

The authors declare that there are no conflicts of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.imlet.2014.11.014.

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